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Expression of Insulin-Like Growth Factor Receptor, IGF-1, and IGF-2 in Primary and Metastatic Osteosarcoma

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Background and Objectives: We have previously shown that insulin-like growth factor (IGF)-responsive murine sarcomas demonstrate inhibition of local and metastatic disease growth when implanted in an IGF-deficient host animal. In this experiment, we tested whether IGF receptor (IGF-R) and ligands were expressed in human primary and metastatic osteosarcomas.

Methods: Fifty-two specimens of human osteosarcoma tumor from 48 patients were assayed for IGF-R, IGF-1, and IGF-2 using reverse transcriptase polymerase chain reaction.

Results: Twenty-one of 46 tumors analyzed had levels of expression of IGF-R greater than or equal to the positive control cell line. Twenty-seven of 44 expressed levels of IGF-1 greater than or equal to the positive control, as did 21 of 38 cases assayed for IGF-2. No differences were found between 40 primary tumor samples and 12 metastatic lesions in mean levels of IGF-R, IGF-1, or IGF-2. There was a moderately strong correlation between expression of IGF-R and IGF-1, suggesting that autocrine stimulation may be an important mechanism for stimulation of osteosarcoma proliferation.

Conclusions: A significant proportion of osteosarcoma tumors express IGF-R and ligands. Higher levels of expression were not correlated with metastatic lesions. *J. Surg. Oncol. 1998;69:21–27.*

KEY WORDS: osteosarcoma; insulin-like growth factor; sarcoma; insulin-like growth factor receptor; bone neoplasms

INTRODUCTION

Despite the use of multidrug adjuvant chemotherapy protocols, a significant proportion of patients with osteosarcoma develop metastatic disease either during or, more commonly, after the completion of drug treatment [1]. Once diagnosed with metastases, the potential for cure in osteosarcoma is markedly decreased [1]. Because most patients developing relapse have already received maximal chemotherapy treatment, it is important to consider new therapeutic approaches for osteosarcoma that do not rely on cytotoxic drugs. Understanding the factors that stimulate osteosarcoma growth and metastasis may be important for developing these novel treatment protocols.

It is evident that insulin like growth factor (IGF) is important for the proliferation of some human malignan-

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cies including sarcomas [2–5]. The IGF signal transduction system involves ligands, receptors, and binding proteins [6–9] and has been shown to be mitogenic for malignant cell lines of both epithelial and mesenchymal origin [10–15]. IGF-1 is a small peptide that mediates the physiological effects of growth hormone (GH) on skeletal growth during the adolescent growth spurt [16,17]. IGF-2 has an important physiological role in fetal and neonatal-natal growth, but is rarely expressed in adult tissues [8]. Both of these ligands exert their effect on cell proliferation and differentiation by binding to the IGF-1 receptor [18], which is an important co-factor in the transformation of cells to a malignant phenotype [2].

It has been shown that nontransformed mesenchymal cell lines will grow in serum-free medium when transfected with IGF-receptor (IGF-R) DNA [19]. Other investigations using cell lines derived from mouse embryos with a targeted disruption in the IGF-R gene have shown that these cells are resistant to transformation with the SV40 T antigen, papillomavirus, or an amplified platelet-derived growth factor receptor [20–22]. A series of experiments using antisense strategies against IGF-R RNA have shown inhibition of soft agar growth in a variety of human cancer cell lines [23,24]. In addition to the effect of IGF signaling in the growth of transformed cells, it has been shown that IGF-2 stimulates motility in sarcoma cells in vitro [10].

We have previously shown that a proportion of human sarcomas express IGF-R and IGF-1 and IGF-2 ligands [25] and that sarcoma cell lines are responsive to IGF in vitro [14]. We have observed using IGF-responsive murine tumors in vivo that both local tumor growth and metastasis can be decreased by lowering serum levels of IGF using surgical hypophysectomy [26,27] or by pharmaceutical agents that block GH production by the pituitary [28]. Furthermore, we have demonstrated that the effect of hypophysectomy on metastasis in IGF-responsive murine tumors can be partially reversed by the administration of human GH to the hypophysectomized host [27]. In similar experiments we have demonstrated that the growth of IGF-responsive murine breast tumors can be inhibited by implantation in mice that are homozygous for lit, a missense gene mutation resulting in loss of function of the pituitary GH-releasing hormone receptor and subsequent suppression of serum IGF-1 levels [29]. These results suggest that some human sarcomas are responsive to IGF mitogenic signals and that alteration of IGF physiology by a variety of therapeutic interventions may offer a low-morbidity method for inhibition of sarcoma metastatic potential in the clinical setting.

In light of these observations, we have questioned what proportion of human osteosarcomas express biologically significant levels of IGF ligand and IGF-1 receptor and whether metastatic osteosarcoma tumors express higher levels of ligand or receptor than primary tumors. The experiments reported below confirm our original finding that both ligands and receptor are produced by a high proportion of human osteosarcomas. However, expression was not increased in samples obtained from lung metastases when compared with samples obtained from primary tumors.

MATERIALS AND METHODS

Tumor Specimens

Samples from surgically resected primary and metastatic osteosarcoma were obtained from viable regions of the tumor and immediately flash frozen in liquid nitrogen. Frozen sections taken from adjacent regions of the tumor were used to confirm the presence of viable tumor. Specimens were obtained from 48 patients in total. In four patients samples from both the primary tumor and the subsequent metastasis were available for analysis. In 36 cases, sample was available only from the primary tumor, and in 8 cases material was available only from the metastatic lesion.

Control Cell Lines

The MCF-7 breast cancer line was used as a positive control for IGF-R since this cell line is responsive to IGF-1 in vitro and demonstrates inhibited growth when implanted in the scid/lit murine host deficient for GH and IGF [29,30]. RPMI 7666 and NCI H69 cell lines were used as positive controls for IGF-1 and IGF-2, respectively, since they have been shown in vitro to produce physiological levels of ligand in conditioned medium [31]. MCF-7 and NCI H69 were maintained in modified Eagle's medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), and RPMI 7666 was maintained in RPMI 1640 and 20% FCS.

RNA Extraction From Specimens and Cell Lines

A portion of each specimen was crushed in an homogenizer while frozen and placed in guanidium thiocyanate. RNA was extracted by ultracentrifugation of nucleic acid through a CsCl gradient as previously described [25]. Cell line RNA was obtained after suspension of monolayers in guanidium thiocyanate. After removal of protein by phenol-chloroform extraction, the sample was ethanol precipitated and resuspended in diethyl pyrocarbonate-treated water.

Quantification of RT-PCR Products

Primers specific for the IGF-1 receptor, IGF-1, and IGF-2 have been described previously [25]. The sense IGF-R R1 primer (5′-ACCCGGAGTACTTCAGCGCT-3′) corresponded to nucleotides 2,975–2,994 in exon 14. The antisense primer R2 (5′-CACAGAAGCTTCGTT-GAGAA-3′) corresponded to nucleotides 3,185–3,204 in exon 16.

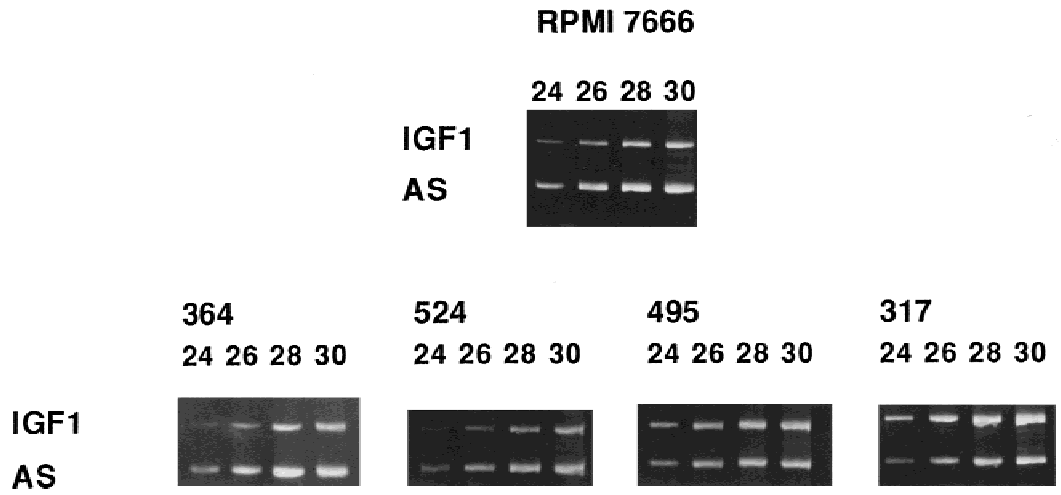

Fig. 1. IGF-1 expression in osteosarcoma. Gel electrophoresis results for control RPMI 7666 cell lines and four tumor samples (364, 524, 495, and 317) analyzed for IGF-1. Cycle numbers for the polymerase chain reaction are evident at the top of the gel. Quantitation by laser densitometry showed that tumors 364 and 524 expressed IGF-1 at lower levels of expression than the control, whereas 495 and 317 expressed the ligand at higher levels.

The sense primer for IGF-1, IG1 (5′-TCTTGAAGGTGAAGATGCACACCA-3′), corresponded to nucleotides 335–342 in exon 1 and 1–6 in exon 2. The anti-sense primer IG2 (5′-AGCGAGCTGACTTGGCAGGCTTGA-3′) corresponded to nucleotides 17–40 in exon 3. The sense primer for IGF-2, IF1 (5′-CTGGTGGACACCCTCCAGTTC-3′), corresponded to sequence 109–129 in exon 5, whereas that of IF2 (5′-GCCCACGGGGTATCTGGGGAA-3′) corresponded to sequence 322–333 in exon 6.

Expression of asparagine synthetase provided an internal control for the amount of RNA template in each polymerase chain reaction (PCR) reaction. Primer A1 (5′-ACATTGAAGCACTCCGCGAC-3′) corresponded to nucleotides 674–793 in exon 4. Anti-sense primer A3 (5′-AGAGTGGCAGCAACCAAGCT-3′) corresponded to sequences 968–986 in exon 6. Anti-sense primer A4 (5′-CCTGAGGTTCTTCACAG-3′) corresponded to sequences 867–886 in exon 5. Asparagine primers A1 and A3 provided a control for the PCR for IGF-1 receptor and IGF-2. Primers A3 and A4 were used as a control for IGF-1. PCR was performed as described previously [25]. All samples were analyzed at least in triplicate, and an arithmetic mean was used to calculate the representative values.

The kinetics of the various reactions were evaluated using the control cell line c-DNA to determine the optimal cycle number to which cDNA products should be co-amplified, while ensuring that production of the PCR products of interest (IGF-1 R, IGF-1, and IGF-2) as well as the internal control AS remained exponential [25]. PCR yields in the control cell lines were plotted on semilogarithmic graphs to determine the appropriate cycle numbers for evaluation of relative levels of expression in patient samples. Each assay was carried out at three different cycle numbers from 22 to 28 cycles to ensure a linear range for the tumor analyzed. Since the quantity of amplified AS product is assumed to be proportional to the amount of initial mRNA template, a relative amount of IGF-R, IGF-1, and IGF-2 product could be determined by normalizing IGF amounts to AS, as determined on laser densitometry quantification of PCR products following acrylamide gel electrophoresis. Finally, relative amounts of IGF-R, IGF-1, and IGF-2 were normalized to the levels found in cell line controls (MCF-7, RPMI 7666, and NCI H69, respectively) (Figs. 1, 2).

Statistical Methods

Comparison of expression of IGF-R, IGF-1, and IGF-2 in primary and metastatic tumors was performed using the Wilcoxon signed rank test. Correlation between IGF-R, IGF-1, and IGF-2 expression was evaluated using the Spearman correlation coefficients.

RESULTS

As shown in Table I, samples were available from 48 patients. In 44 cases, only single time point specimens were available for analysis. Thirty-six of these single specimens were derived from a primary tumor, and eight specimens were obtained from metastases for which no primary tumor was available.

In four cases, specimens were obtained from the primary tumor and from the metastatic lesion. Overall, 52 specimens were available from 48 patients. In total, 40 samples were obtained from primary tumors, and 12 specimens were taken from metastases.

Expression of IGF-1 R, IGF-1, and IGF-2 was detected in each tumor tested. As seen in Table I, relative expression for IGF-1 R varied over more than a 20-fold

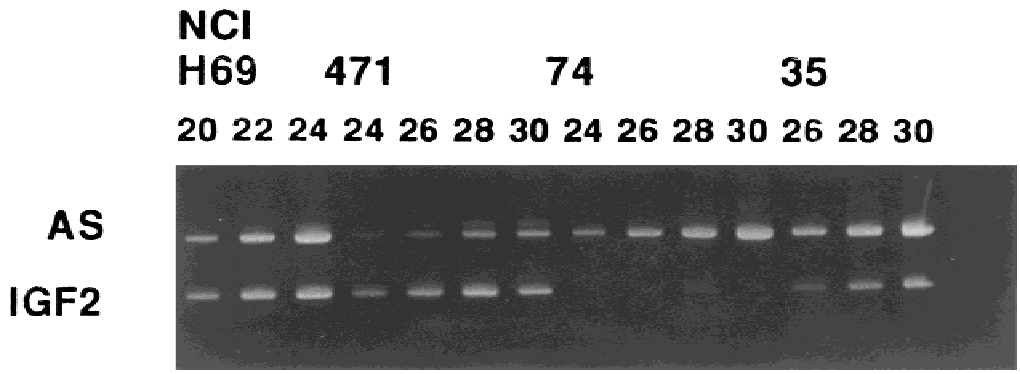

Fig. 2. IGF-2 expression in osteosarcoma. Gel electrophoresis results for NCI H69 control line and three tumors (471, 74, and 35) analyzed for IGF-2. Quantitation by laser densitometry showed low expression in tumors 34 and 35 relative to the control cell line and higher levels of IGF-2 expression in 471.

range, from a minimum of 0.2 to a high of 5.2 (with a comparative level of expression equaling 1 for the positive cell line, MCF-7). IGF-1 relative expression varied over a similar range, from a minimum value of 0.2 to a maximum of 4.6 (relative to a value of 1 for RPMI 7666). IGF-2 variation was similar, from a minimum value of 0.1 to a maximum value of 3.1 (relative to the control cell line NCI H69).

Comparison of IGF-R, IGF-1, and IGF-2 did not differ significantly between primary and metastatic lung lesions. The mean value for IGF-R expression in primary lesions was 1.1 and in metastases was 1.3 ($P = 0.74$, Wilcoxon signed rank test). In primary tumors, IGF-1 expression averaged 1.4, compared with 1.0 in metastases ($P = 0.9$). Comparable mean values for IGF-2 were 0.7 in primary osteosarcoma and 0.7 in metastases ($P = 0.3$).

In the four cases, (#s 3, 11, 14, and 15), for which both primary and metastatic lesions were available, no consistent changes in patterns of expression were found from primary to metastatic tumor (Table I). In case 3, IGF-R was estimated as 1.6 in the biopsy sample and 2.3 in the metastasis. IGF-1 was 2.5 in the primary and 2.0 in the metastasis. IGF-2 was not analyzed in the metastasis.

For case 11, both IGF-R and IGF-1 increased from the primary to the metastasis (0.5 to 1.5 for IGF-R and 1.0 to 2.0 for IGF-1), whereas IGF-2 decreased (1.5 to 0.3). In case 14, IGF-R decreased from biopsy to metastasis (5.3 to 2.7), whereas IGF-1 increased (1.5 to 3) and IGF-2 decreased (1.6 to .0.5). Finally in case 15, IGF-R and IGF-2 remained consistent (0.9 to 1.0 for (IGF-R, and 1.6 to 1.7 for IGF-2) in the primary and metastasis, whereas IGF-1 increased somewhat (1.8 to 3.3). The only notable trend in the four tumors analyzed was increased expression of IGF-1 in three tumors from the initial to the metastatic sample.

Evaluating the potential for autocrine and paracrine stimulation of proliferation in the samples analyzed, 21 of 46 tumor specimens had IGF-R levels of expression equal to or greater than MCF-7 cell lines. Twenty-seven of 44 specimens had IGF-1 expression equal to or greater than the RPMI 7666 cell lines, and 21 of 38 samples had levels of IGF-2 expression equal to or greater than the NCI H69 cell line. There was a modest correlation between the level of expression of IGF-R and IGF-1 (0.38, Spearman correlation coefficient), but no evident correlation between IGF-R and IGF-2 (0.06, Spearman correlation coefficient).

DISCUSSION

The outcome of osteosarcoma management has improved substantially with the advent of adjuvant and neoadjuvant chemotherapy. A randomized trial of the efficacy of chemotherapy in the management of this sarcoma demonstrated a definite survival advantage for patients managed with adjuvant drug treatment that has persisted at long-term follow-up [32,33]. Despite the benefit of chemotherapy, however, approximately 30–40% of patients who present with localized disease will develop lung metastases despite cytotoxic therapy [1]. Since these patients already receive multiagent chemotherapy at maximally tolerated doses, it is unlikely that further improvement in survival will be achieved by intensifying current drug treatment. In developing novel therapeutic approaches, it is reasonable to determine whether biological factors that stimulate osteosarcoma proliferation and metastasis can be identified and modified to achieve a clinical advantage.

There is strong empirical evidence that the IGF signal transduction pathway may be important in osteosarcoma. The highest incidence of osteosarcoma is found in the second decade of life at a time when adolescent skeletal growth acceleration is induced by increased production of GH and elevated serum levels of IGF [34–36]. The most common anatomic sites for development of osteosarcoma are those that respond to this increase in serum levels of IGF with the maximal epiphyseal growth re-

TABLE I. IGF Receptor and Ligand Expression in Primary and Metastatic Osteosarcoma*

Case no.	Site	IGF-R	IGF-1	IGF-2
1	Primary	0.80	0.18	0.58
2	Primary	0.78	0.22	—
3	Primary	1.62	2.54	—
	Metastatic	2.25	2.04	—
4	Metastatic	0.22	0.40	0.42
5	Metastatic	0.92	1.17	1.06
6	Primary	1.66	1.07	1.02
	Metastatic	1.02	2.12	—
7	Primary	0.34	—	—
8	Primary	0.08	2.82	0.55
9	Primary	—	3.11	1.54
10	Metastatic	1.27	2.38	1.13
11	Primary	0.49	0.98	1.56
	Metastatic	1.46	2.06	0.31
12	Primary	1.40	—	—
13	Primary	1.10	1.08	1.03
14	Primary	5.27	1.25	1.56
	Metastatic	2.74	3.05	0.46
15	Primary	0.86	1.75	1.62
	Metastatis	0.97	3.30	1.80
16	Metastatic	1.20	0.34	1.52
17	Metastatic	1.08	0.83	—
18	Primary	0.73	0.95	0.33
19	Primary	1.42	0.75	0.57
20	Metastatic	1.11	0.52	0.09
21	Primary	1.47	4.61	1.08
22	Metastatic	1.09	0.68	0.24
23	Primary	1.27	2.28	—
24	Primary	1.07	2.59	2.22
25	Primary	0.49	0.81	1.16
26	Primary	0.89	4.53	1.58
27	Primary	0.38	—	—
28	Primary	0.88	1.41	0.55
29	Primary	0.72	0.88	0.72
30	Primary	0.23	1.81	
31	Primary	0.43	—	—
32	Primary	0.64	0.78	1.04
33	Primary	0.65	0.68	1.26
34	Primary	0.80	—	—
35	Primary	—	4.20	—
36	Primary	1.32	—	—
37	Primary	1.44	1.35	—
38	Primary	0.54	1.28	0.79
39	Primary	1.24	1.24	1.53
40	Primary	0.76	0.80	2.49
41	Primary	0.67	0.73	0.19
42	Primary	0.74	3.97	1.52
43	Metastatic	0.82	0.97	0.33
44	Primary	—	1.70	—
45	Primary	—	2.09	0.36
46	Primary	—	—	0.73
47	Primary	—	—	3.10
48	Primary	—	—	0.88

*All numbers represent ratio of densitometry readings of specimen to control cell lines (normalized as described in text).

sponse (i.e., the distal femur and the proximal tibia) [36]. Other investigators have suggested that osteosarcoma patients have higher serum levels of IGF than the general population [37]. Our group has been interested in determining whether alteration in IGF physiology can have an effect on the biological behavior of osteosarcoma.

We have previously shown that osteosarcoma cell lines demonstrate a proliferative response to IGF in vitro [14]. We have quantitated IGF-R in three murine sarcoma models and have shown that the tumors with IGF-R levels equal to or greater than MCF-7 demonstrate inhibited local tumor growth when implanted in mice who have undergone hypophysectomy to lower serum IGF levels [26,27]. We have shown in two different murine sarcomas that hypophysectomy decreases the development of metastases in the host [26,27]. Furthermore, the effect of hypophysectomy in decreasing metastatic potential was partially reversed in the RIF murine sarcoma model by treating the hypophysectomized animals with human GH [27]. We have also duplicated the effect of hypophysectomy in the RIF model by treating animals with medication that suppresses GH without resorting to hypophysectomy [28]. Similar results in decreasing sarcoma growth in vivo using pharmaceutical methods to lower serum IGF have been observed by Pinski et al. [38] using human osteosarcoma cell lines implanted in immunosuppressed mice.

These results suggest that modification of the IGF system in vivo may offer promise as a nontoxic adjuvant therapy for osteosarcoma. In developing this potential therapy, it is important to determine the proportion of human tumors that may be IGF responsive. We have previously tested 29 human sarcomas (including 10 primary osteosarcomas) for IGF-1 R, IGF-1, and IGF-2 expression using reverse transcriptase (RT)-PCR [25]. In the current experiment we have extended this initial series by analysis of a further 52 osteosarcoma samples taken from 48 patients and have questioned whether IGF receptor or ligand is expressed at higher levels in metastatic lesions than in primary tumors. It would be reasonable to assume that metastasizing clones of cells might develop the phenotype associated with metastasis by increasing endocrine or autocrine responsiveness to IGF. However, as shown in the results reported above, we were unable to identify differences between primary and metastatic tumors in levels of expression of either receptor or ligand in these experiments.

To provide a reference level for the biological significance of the RT-PCR results, we have utilized cell lines that have been tested for IGF responsiveness and IGF ligand production in vitro and in vivo. MCF-7, for example, is known to proliferate in vitro in response to small quantities of IGF-1 [30]. Conditioned medium from both RPMI 7666 and NCI H69 is known to cause MCF-7 proliferation, and IGF production from these cell lines has been quantitated in vitro [31]. Finally, our group has recently shown that MCF-7 cells, implanted in scid/lit mice deficient for GH and IGF, grow more slowly than in control animals [29]. These in vivo results con-

firm the in vitro data. It would therefore be reasonable to expect that tumors with IGF-1 receptor mRNA transcripts equal to or greater in number than MCF-7 would be IGF responsive and that tumors with IGF-1 or IGF-2 levels of expression equal to or greater than the RPMI 7666 and NCI H69 control cell lines might be capable of autocrine stimulation.

In determining whether the results of IGF-R RT-PCR adequately reflect IGF-R protein quantitation, we previously compared the results of RT-PCR with competitive binding studies and Scatchard plot analysis in 25 human sarcomas (including six osteosarcomas). The amount of material necessary for membrane extraction of protein and competitive binding makes this method impossible for most of our clinical osteosarcoma specimens. However, in the clinical specimens tested, a moderate to high correlation between the two methods was observed [25]. We are therefore satisfied that the methods used above are adequate (given the small amounts of tumor available for analysis) for quantitation of IGF-R and ligands.

The results described above suggest that approximately 50% of osteosarcomas are responsive to IGF and that more than 50% of osteosarcomas produce levels of ligand capable of inducing an autocrine stimulation of tumor proliferation. Furthermore, the correlation of IGF-R and IGF-1 expression suggests that a substantial proportion of tumors may be self-sufficient in providing both ligand and receptor. Despite the seeming importance of the IGF signal transduction pathway in osteosarcoma, there was no suggestion that ligand or receptor are expressed at a higher level in metastases than in primary tumors.

These results have significance for the design of clinical methods to alter osteosarcoma growth and metastasis through the IGF pathway. Other investigators have found similar results in giant cell tumors of bone [39] and in Ewing sarcoma [40]. At present there are several potential methods for altering serum IGF, either surgically (by hypophysectomy) or pharmacologically. However, the efficacy of lowering serum IGF may be decreased if the tumor is capable of autocrine stimulation through the production of ligands. It is possible that lowering of the background level of IGF ligand in the interstitial fluid by lowering serum IGF will be effective in decreasing the effectiveness of IGF produced by the tumor. It would certainly be more effective, however, to provide direct inhibition of the IGF-1 receptor in designing pharmacological strategies for IGF inhibition.

The finding that IGF ligand and receptor is not expressed at increased levels in metastatic lesions may suggest that the IGF tyrosine kinase signal transduction system is maximally stimulated by the levels of IGF activity seen in primary tumors and that there is no competitive advantage offered by further activation through this system. Indeed, the fact that 5 of the 12 lung metastases analyzed expressed IGF-R at levels lower than MCF-7 suggests that metastases can occur without maximal IGF stimulation and that another growth factor pathway is probably responsible for proliferation in a proportion of osteosarcomas.

These results confirm, however, that about 50% of osteosarcomas may be appropriate for trials of IGF-modifying therapy. The development of new pharmacological agents and the availability of transgenic mice with deficient serum IGF should be useful in determining which osteosarcomas may be most appropriate for novel therapies involving alteration of IGF physiology.

CONCLUSIONS

Approximately 50% of osteosarcoma tumors express IGF-R at high levels, and about 50% of tumors express high levels of IGF-1 or IGF-2 ligands.

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